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(54) Title: MONOCLONAL ANTIBODIES SPECIFIC FOR ACETYL CHOLINESTERASE

(57) Abstract

A monoclonal antibody which is specific for human secretory acetylcholinesterase (sAChE) and which shows no cross-reactivity with other types of human acetyl cholinesterase is useful for determining human sAChE in a sample and consequently for detecting Neural Tube Defects such as anencephaly, encephalocele and spina bifida.

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# MONOCLONAL ANTIBODIES SPECIFIC FOR ACETYL CHOLINESTERASE

This invention relates to monoclonal antibodies, to their preparation and to their use in immunoassays.

Neural Tube Defects (NTDs) are a number of structural anomalies of the central nervous system (CNS) of the human fetus. They result from the failure of the neural tube to close properly during embryogenesis. There are three major types of NTD:

(1) Anencephaly: where the closure defect can occur at the top of the neural tube. It results in babies being stillborn.

(2) Encephalocele: is relatively uncommon, with the closure defect occurring a little lower down the neuroaxis. Babies do survive with little paralysis, and they usually grow up spastic, severely retarded and often blind.

(3) Spina bifida: where the closure defect occurs lower still resulting in babies being born with a portion of the spinal cord exposed. This results in paralysis of the legs, incontinence and hip, knee and feet deformities. About 90% of these babies will die if not operated on.

NTD incidence varies considerably throughout the world. In Europe, the prevalence is 6-13 per 10,000 births. The risk goes up considerably in women over 35 years of age. Currently NTD's are diagnosed by:

(1) Genetic counselling: by checking the family history to find out if a couple is in a risk group, i.e. if they have a previously affected child or parent or close relative. A drawback of this method is that over 95% of all NTDs occur in couples not previously considered at risk from this disorder.

(2) Ultra-sound scanning (US): has proved very useful in identifying many fetal abnormalities. However, there are some drawbacks:

(a) US has 100% accuracy in detecting anencephaly but only 80% for spina bifida.

(b) It relies on the experience of the operator f th scanner. The risk of missing malformation is estimat d at 1.6%.

(c) Misdiagnosis occurs if scanning is done at a very early gestational age.

(3) Alpha-fetoprotein (AFP): is a glycoprotein with a molecular weight of 70,000 Daltons. It is usually secreted by the embryo yolk sac and later by the fetal liver. It diffuses into the Amniotic Fluid (AF) and then into the maternal circulation. AFP levels were found to be elevated in maternal serum of women with NTD fetuses. Currently, its measurement by an immunoassay is the main diagnostic test, in conjunction with US, for NTD.

Normally, AFP is measured in maternal serum. An elevated value is followed by US scanning, then by amniocentesis. The amniotic fluid (AF) is then tested for AFP. However, there are problems with AFP:

(a) It requires a precise knowledge of gestational age since the normal levels of AFP vary in maternal serum considerably with gestational age.

(b) AFP levels in AF also vary with gestational age. Furthermore, a major problem with assaying AFP in AF is the likelihood of a false positive due to contamination of the AF samples with fetal blood and/or maternal blood.

A U.K. collaborative study has found that only 80% of NTD are detected by AFP measurements.

(4) Neural-specific acetyl cholinesterase (AChE): since the main defect in NTD is the incomplete closure of the neural tube, many of the neurally-derived proteins and other components are expected to be found in the AF and to diffuse into maternal serum. These could, therefore, be potential markers for NTD. In 1979, Smith et al. (Lancet, i,685,1979) proposed the analysis of AChE in AF as a supplementary test to improve the diagnosis of NTD.

Cholinesterases are a group of enzymes that hydrolyse

cholinesters into cholin . Pseudo-cholinesterases, which are present in serum, muscle and brain, hav a substrate preference to butyryl cholines. Acetyl cholinesterases (AChE) have a substrate preference to acetyl cholines. Th two types are different immunologically and biochemically. AChEs are of multiple forms, globular and asymmetric forms. The latter are found predominantly in muscle tissue. Globular forms are dominant in the central nervous system and red blood cells (RBC). They exist as a monomer, dimer, or tetramer. Globular AChEs are membrane-bound or soluble.

In human brain, about 70% of AChE is membrane-bound and 100% of red blood cells AChE is membrane-bound. The central nervous system soluble AChE is in itself of multi-forms. The bulk of it is believed to be derived from the membrane-bound AChE as is shown by its behaviour in the presence of detergents.

One type of the neural soluble AChE is known as the secretory AChE (sAChE). It is secreted by neural cells, has a distinct electrophoretic mobility, is the only form of AChE found in mammalian cerebrospinal fluid (CSF), is not influenced by detergents and is the only form that binds selectively to the AChE inhibitor edrophonium chloride when this is immobilised on Sepharose (Trade Mark)-4B gel. The isolation and partial characterisation of the secretory form of human brain AChE has been described (Gardner et al., Biochem.Soc.Trans., 14,1234-1235,1986).

In amniotic fluid (AF), there are three forms of AChE present in addition to pseudo-ChE. A monomer (4.0 S), a dimer (5.5 S), and a tetramer (10.3 S). The origin of the first two is not clear. However, the dimer is very much similar to the dimer membrane-bound AChE found in red blood cells (RBC). The tetramer form is the neural-specific form, the secretory AChE (sAChE). The amount of this form goes up to 62-fold in NTD.

AChE is detected in AF in three ways. These are:

(a) Enzymatically: by using the standard Ellman test for assaying esterases. The test requires the use of highly specific inhibitors in order to be able to distinguish between AChE and pseudo-ChE. It can not distinguish between the different forms of AChE. Hence, it is useless in cases where the AF samples are contaminated by fetal or maternal blood.

A recent modification to this test involves a prior separation of the different molecular forms by sucrose density gradient centrifugation followed by the Ellman test. This makes the test complicated requiring ultra-centrifuges, and overnight spins. Furthermore, it does not distinguish between the sAChE and other brain-derived AChE with similar molecular weight.

(b) Gel electrophoresis and histochemical staining: this is the standard procedure currently employed in clinical laboratories. It relies on the electrophoretic mobility of the sAChE. AF samples are electrophoresed on 6% acrylamide gels which are subsequently stained for esterase activity. Specific AChE activity is identified by using a specific inhibitor. A U.K. collaborative study has shown this test to be very useful, finding that AChE levels do not vary with gestational age as in the case with AFP. Therefore, precise knowledge of fetal age is not necessary.

There are a number of drawbacks with this procedure, though:

(i) It is a qualitative test.

(ii) The test is useless if AF samples are contaminated with fetal or maternal blood because serum pseudo-ChE and RBC AChE mask the specific sAChE band on the gel.

(iii) The test is time-consuming as it requires electrophoresis and histochemical staining.

(c) Immunological testing: many different antibodies, both polyclonal and monoclonal, have been raised against

different forms of AChE. Many of these do not cross react with pseudo-ChE. Zaneta et al (FEBS Lett.129,293,1981) raised a rabbit antiserum against rat brain membrane AChE, which had only low cross reactivity with soluble AChE. Gennari and Brodbeck (J. Neurochem. 44,697,1985) reported an antiserum raised to detergent soluble AChE from human brain which showed a moderate preference for detergent soluble versus the water soluble enzyme. An antibody raised to rabbit AChE binds the membrane-bound form of brain AChE better than the soluble form (Rakonczay and Brimijoin, BBA 832, 127,1985).

Immunological differences between AChEs of different tissues are also coming to light. Most antibodies raised to AChE show equivalent affinity for brain and erythrocyte enzymes from the same species. Rasmussen et al (Clin.Chim. Acta,166,17,1987) evaluated 11 monoclonal antibodies raised to human RBC AChE with respect to reactivity with AChE from both RBC and brain. They claimed that one of their monoclonals actually showed preference for membrane-bound AChE purified from human brain, but all their monoclonal antibodies reacted with RBC AChE.

There are few reports on the use of immunoassays of AChE in the diagnosis of NTD. The main problems are the difficulty of obtaining specificity to the neural and more exactly, the secretory AChE. Reported assays rely on the use of either monoclonal or polyclonal antisera raised to human brain AChE. The assays are of two main types.

In the first type of assay, the antigen, AChE, is sandwiched between two monoclonal or one monoclonal and one polyclonal antibodies (Brimijoin et al., J. Neurochem.49, 555,1987). The second type is also of the antigen-capture type but relies on the AChE's own enzyme activity. Monoclonal anti-RBC AChE antibodies are immobilised followed by incubation with test sample. Bound AChE is detected by using the Ellman test (Norgaard et al,

Chin.Chim.29,1061,1983). This second approach does not require pure AChE. Varying degree of success have been reported for these assays when used to diagnos NTD (Norgaard et al.,1983; Brock and Bader,Clin.Chim.Acta 127, 419,1983; Brimijoin et al, Fed.Proc.46,2557,1987; Brock et al., Lancet i,5,1985; Sorensen et al., Prenat.Diag 7,75, 1987).

There are a number of problems with immunoassays of AChE in the diagnosis of NTD:

(i) Scarcity of material has meant difficulty in raising antisera to neural AChE.

(ii) Currently available antibodies are raised to RBC AChE and membrane-bound AChE. They are useless in detecting AChE in AF if the AF is contaminated with fetal or maternal blood, due to the relatively huge amounts of RBC AChE present in such samples.

(iii) Although it is not entirely clear, all the current antisera are thought to react with many types of AChE. They are not specific for the sAChE or at least to the brain-derived AChE.

Measurement of AChE in maternal serum has often been proposed as a test for the prenatal diagnosis of NTDs. Such a test would make it possible to avoid doing amniocentesis with all the risks to the fetus involved in this procedure. However, such a test is not possible yet because of the presence of pseudo-ChE and a number of AChE forms in the serum, as already mentioned.

Fetal and adult human sAChE have now been purified. Using these and special techniques, immortalised cell lines have been obtained which secrete monoclonal antibody specific for human sAChE. Standard in vivo immunisation methods of preparing hybridomas did not result in cell lines which secrete human sAChE-specific antibody. Specific screening strategies were adopted in order to detect hybridomas producing the monoclonal antibody

specific for human sAcheE. The monoclonal antibody can be used in immunoassays for fetal and adult human sAChE, thus enabling NTDs to be detected.

Accordingly, the present invention provides a monoclonal antibody which is specific for human sAChE and which shows no cross-reactivity with other types of human acetylcholinesterase. The monoclonal antibody may be IgG or IgM. It may be rat,mouse or human monoclonal antibody. The monoclonal antibody is prepared by a process which comprises:

(a) culturing an immortalised cell line which secretes the monoclonal antibody; and

(b) isolating the monoclonal antibody thus produced.

The invention also provides an immortalised cell line which secretes a monoclonal antibody specific for human sAChE. Such a cell line can be prepared by one of four processes:

Process (I) comprises:

(a) immunising an animal with adult or fetal human sAChE;

(b) obtaining lymphatic tissue cells from the immunised animal;

(c) culturing the obtained cells with adult or fetal human sAChE;

(d) immortalising the cultured cells; and

(e) screening the resulting cell lines for an immortalised cell line which secretes monoclonal antibody which is specific for human sAChE.

Process (II) comprises:

(a) obtaining lymphatic tissue cells from an animal,

(b) culturing the obtained cells with adult or fetal human sAChE,

(c) immortalising the cultured cells, and

(d) screening the resulting cell lines for an immortalised c ll lin which secretes monoclonal antibody

which is specific for human sAChE.

Process (III) comprises:

(a) obtaining lymphatic tissue cells from an animal,

(b) culturing the obtained cells with adult or fetal human sAChE,

(c) culturing the cells obtained in step (b) with fresh adult or fetal human sAChE,

(d) immortalising the cells obtained in step (c), and

(e) screening the resulting cell lines for an immortalised cell line which secretes monoclonal antibody which is specific for human sAChE.

Process (IV) comprises:

(a) immunising an animal with adult or fetal human sAChE by inoculation directly into a lymphatic organ or lymph node,

(b) obtaining lymphatic tissue cells from the immunised animal,

(c) immortalising the cells obtained, and

(d) screening the resulting cell lines for an immortalised cell line which secretes monoclonal antibody which is specific for human sAChE.

Typically, the same type of human sAChE, adult or fetal, is used in both step (a) and step (c) of process (I) and in both step (b) and step (c) of process (III). In one embodiment, splenocytes are obtained in step (b) of process (I) or (IV) or in step (a) of process (II) or (III). Preferably the cultured cells are fused with myeloma cells in step (d) of process (I) or (III) or in step (c) of process (II) or (IV). Generally resulting cell lines are screened by

- contacting a solid support coated with adult or fetal human sAChE with supernatant from each cell line culture and with, in the presence of polyethylene glycol, a labelled antibody capable of binding to any monoclonal antibody in the supernatant which binds to th human sAChE;

or

- contacting a solid support coated with adult or fetal human sAChE with supernatant from a culture of each cell line grown in serum-free or low serum-supplemented medium and with a labelled antibody capable of binding to any monoclonal antibody in the supernatant which binds to the human sAChE.

The invention further provides a method of determining human sAChE in a sample, which method comprises carrying out a said determination using a monoclonal antibody which is specific for human sAChE and which shows no cross-reactivity with other types of human acetylcholinesterase. The method may comprise contacting a sample suspected of containing fetal human sAChE with a said monoclonal antibody and determining whether the said monoclonal antibody has bound to any fetal human sAChE.

The invention additional provides a test kit suitable for use in determining fetal human sAChE, which kit comprises a monoclonal antibody which is specific for fetal human sAChE and which shows no cross-reactivity with other types of human acetylcholinesterase and means for determining whether the monoclonal antibody has, in use, bound to human sAChE.

The monoclonal antibodies are specific for human sAChE. They react specifically with adult or fetal human sAChE and show no cross-reactivity with other types of human acetylcholinesterase. In particular, they show no reactivity with human red blood cell AChE, and preferably no cross-reactivity with human serum AChE, human membrane-bound neuronal AChE, human muscle AChE and neuronal-soluble non-secretory AChE. The antibodies do not cross-react either with human serum pseudo-ChE. If raised to fetal sAChE, the monoclonal antibodies can bind preferentially to fetal human sAChE rather than to adult human sAChE.

Immortalised cell lines which secrete such monoclonal

antibodies require purified human sAChE for their preparation. This purified material is obtained from human brains. Fetal human brains ar typically obtain d from aborted fetuses over 16 weeks old. The brains are typically stored frozen, thawed and homogenised in ice-cold 0.3M sucrose containing 2mM ethylene diamine tetraacetic acid (EDTA) or using phosphate buffered saline/2mM EDTA as the buffer. The homogenate may be subjected to one or more cycles of freezing and thawing, and then centrifuged. The supernatant is removed and optionally is subjected to gel filtration. Sephacryl S-200 can be used for this purpose. Fractions are collected having a protein content as determined by optical density at 280 nm, and AChE activity as determined by the Ellman assay.

The sAChE in the supernatant from centrifugation or in the fractions collected during gel filtration is then purified by affinity chromatography. A column of edrophonium chloride-epoxy-Sepharose gel may be used. Bound material is eluted, from which edrophonium chloride is removed by gel filtration. Fractions containing AChE activity are pooled and lyophilised. They can be reconstituted in phosphate-buffered saline and frozen for storage. In this way human sAChE having an activity of 900-1500 units/mg may be obtained.

An immortalised cell line may be prepared by any one of process (I) to (IV). Prior to immortalisation:

Process (I). A non-human mammalian host such as a mouse or rat is inoculated with the adult or fetal human sAChE. After a sufficient time has elapsed to enable the host to mount an antibody response to the protein, antibody-producing cells are removed from the host. Cells of lymphoid origin such as splenocytes, lymph node cells or other lymphatic tissue or peripheral blood lymphocytes may be obtained from the immunised host.

Process (II). Cells of lymphoid origin such as

splenocytes, lymph node cells or other lymphatic tissue or peripheral blend lymphocytes may be obtained from a mammalian host such as a mouse or rat. The cells are incubated in vitro in a culture medium containing adult or fetal human sAChE. Typically 5 x $10^6$ cells/ml are incubated with 0.8 µg/ml of sAChE in 15ml of culture medium in a 25 $cm^2$ flask. The culture medium may also contain thymocyte-conditioned medium, culture supernatant from EL4 cells, phorbol myristate acetate or other growth factors. Typically the incubation lasts for a minimum of 3 days, for example 4-10 days. A suitable period may be 5 days or 6 days. By increasing the incubation time up to 10 to 14 days in the presence of adjuvant peptides, a class switch from IgM to IgG antibody can be induced. The cultured cells are then immortalised.

Process (III). Cells of lymphoid origin such as splenocytes lymph node cells or other lymphatic tissue or peripheral blood lymphocytes may be obtained from a mammalian host such as a mouse or rat. The cells are incubated in accordance with the procedure for process (II). An in vitro boost may be given by transferring the cells to fresh culture medium containing sAChE. The culture medium may also contain thymocyte-conditioned medium, culture supernatant from EL4 cells, phorbol myristate acetate or other growth factors. Typically this second incubation or any subsequent incubation lasts for a period from several hours to 10 days. A suitable period may be 5 days or 6 days. By increasing the incubation time up to 10 to 14 days in the presence of adjuvant peptides, a class switch from IgM to IgG antibody may be induced. The culture cells are then immortalised.

Process (IV). A non-human mammalian host such as a mouse or rat is immunised with adult or fetal human sAChE by direct inoculation into a lymphatic organ or lymph node. An adjuvant may also be included in the inoculation.

Typically a mouse is anaesthetised with hypnorm/midazolan. The fur is clipped over the spleen and an incision 1-1.5 cm long is made just below the left set of ribs. Th muscle is separated using blunt dissection, and the lower pole of the spleen exteriorized through a small incision in the peritoneum. The sAChE is inoculated directly into the spleen and the skin in stitched. After a sufficient time has elapsed to enable the host to mount an antibody response to the protein, antibody-producing cells are removed from the host. Cells of lymphoid origin such as splenocytes, lymph node cells or other lymphatic tissue or peripheral blend lymphocytes may be obtained from the immunised host. Typically the lymphoid cells removed are splenocytes, from a mouse, between 2-4 days following immunisation. A suitable period may be 3-5 days. The lymphoid cells are then immortalised.

The cells from the immunised host are incubated in vitro in a culture medium containing further adult or fetal human sAChE. The culture medium may contain also thymocyte-conditioned medium or other cell growth factors. Typically the incubation lasts for a minimum of 3 days, for example from 4 to 6 days. A suitable period is 5 days. By increasing the incubation time up to 10 to 14 days in the presence of adjuvant peptides, a class switch from IgM to IgG antibody can be induced.

For cells to be immortalised, they may be fused with cells of an immortalising cell line. This cell line may be a myeloma cell line, e.g. of a mouse or rat. Alternatively, or additionally, the cultured cells may be transformed with, for example, a virus. The resulting fusions are screened. An immortalised cell line, such as a hybridoma, secreting antibody specific for human sAChE is thereby selected.

The fusions are typically screened by an enzyme-linked immunosorbent assay (ELISA). A solid support, such as

microtitre wells, is coated with adult or fetal human sAChE. Supernatant from a cultur of a fusion is incubated with the coated support. A labelled antibody capable of binding to any monoclonal antibody in the supernatant which binds to the human sAChE is added. The labelled antibody is generally added after the coated support has been incubated with the culture supernatant and the support has been washed.

In one embodiment the labelled antibody is incubated with the support in the presence of a polyethylene glycol (PEG). PEG 4000 or 6000 may be added. The amount of PEG may be from 1 to 10% w/v, for example from 2 to 6% w/v and preferably about 4% w/v. In another embodiment, the labelled antibody is incubated with the support in the absence of a PEG provided the supernatant is a supernatant from a cell line which has been grown in, or pulsed with, a serum-free medium or a medium containing 1% v/v or less of serum, typically fetal calf serum. The labelled antibody binds to any monoclonal antibody, originating from the culture supernatant, which has bound to the human sAChE coating the solid support. The label is typically an enzyme label such as horse radish peroxidase. In this way fusions secreting antibody specific for human sAChE can be detected, after addition of a substrate for the enzyme in the case where an enzyme label is employed.

The resulting immortalised cell line can be used to obtain monoclonal antibody specific for human sAChE. Monoclonal antibody is prepared by:

(a) culturing an immortalised cell line which secretes the monoclonal antibody; and

(b) isolating the antibody thus produced.

Progeny of clones producing the desired antibody can be grown. Step (a) may be conducted <u>in vitro</u> in suitable culture media in tissue culture flasks, in a hollow fibre tissue culture device or in a cell fermenter for example.

Alternatively cells may be cultured in step (a) in vivo. They may b grown in vivo in laboratory animals, for example in laboratory animals, such as mice and rats. Cells of the immortalised cell line may be implanted in a body cavity, such as the abdominal cavity, of the animal and allowed to grow. The resulting monoclonal antibody can be separated from the culture medium or from the body cavity fluid such as the ascites fluid of the animal by techniques such as ammonium sulphate precipitation, ion exchange chromatography, gel filtration, affinity chromatography, high-performance liquid chromatography, etc.

The monoclonal antibody can be used in the diagnosis of NTDs and, in particular, anencephaly, encephalocele and spina bifida. The monoclonal antibody can also be used in the diagnosis of omphalocele, gastroschisis, intrauterine death, esophageal atresia, twin pregnancy with acardiac fetus, normal infant, teratoma, ascites, cystic hygroma, hypoplasia of heart and lungs, cloacal exstrophy, hydrocele, epidermolysis bullosa dystrophica, and aplasia cutis congenita; as well as other neurodegenerative disorders such as senile dementia, Parkinson's disease, and Alzheimer's disease. In each case, diagnosis depends upon detecting and measuring levels of sAChE. For the diagnosis of a pre-natal condition preferably a monoclonal antibody is employed which has been raised using fetal sAChE. A monoclonal antibody which binds preferentially to fetal human sA ChE rather than to adult human sAChE can be employed.

Adult or fetal human sAChE can therefore be assayed using the monoclonal antibody. A sample suspected of containing human sAChE is contacted with the monoclonal antibody. The sample may be a biological fluid such as amniotic fluid or maternal serum from a pregnant woman. An assay may be performed qualitatively, semi-quantitatively

or quantitatively.

A variety of assay formats may be employed. The monoclonal antibody can be used to capture adult or fetal human sAChE selectively onto a solid surface from solution, to label selectively this protein or both to capture and to label this protein. The antibody also may be used in a variety of homogeneous assay formats in which the protein is detected in solution with no separation of phases.

The types of assay in which antibody is used to capture the protein from solution involve immobilization of the antibody onto a solid surface. This surface should be capable of being washed. The types of surfaces which may be used include polymers of various types (moulded into microtitre wells; beads; dipsticks; aspiration tips; electrodes; and optical devices), particles (for example latex; stabilized red blood cells (erythrocytes); bacterial or fungal cells; spores; gold or other metallic or metal-containing sols; organic sols; and proteinaceous colloids; with the usual size of the particle being from 0.005 to 5, for example from 0.1 to 5, microns), membranes (for example of nitrocellulose; paper; cellulose acetate; chemically-activated membranes such as Millipore Immobilon (Trade Mark) or Pall Biodyne (Trade Mark); and high porosity/high surface area membranes of an organic or inorganic material).

The attachment of the antibody to the surfaces can be by passive adsorption from a solution of optimum composition which may include surfactants, solvents, salts and/or chaotropes; or by active chemical bonding. Active bonding may be through a variety of reactive or activatable functional groups which may be exposed on the surface (for example condensing agents; active esters; acid halides; anhydrides; amino, hydroxyl, or carboxyl groups; sulphydryl groups; carbonyl groups; diazo groups; unsaturated groups). Immobilisation may be through a preliminary coating with

protein A or antibody to murine immunoglobulin.

After contacting (reacting) the surface bearing the antibody with a test sampl , allowing time for reaction and, where necessary, separating or removing the excess of the sample by any of a variety of means (washing, centrifugation, filtration, application of a magnetic field, capillary action), the captured protein is detected by any means which will give a detectable signal. For example, this may be achieved by use of a labelled molecule, in particular antibody, or particle as described above which will react with the capture protein. Alternatively, the activity of the immobilised AChE can be assayed.

The detectable signal may be optical or radio-active or physico-chemical and may be provided either directly by labelling the molecule or particle, especially antibody, referred to with for example a dye, radiolabel, electroactive species, magnetically resonant species or fluorophore, or indirectly by labelling the molecule or particle with an enzyme itself capable of giving rise to a measurable change of any sort. Alternatively the detectable signal may be due to, for example, agglutination, diffraction effect or birefringent effect occurring if any of the surfaces referred to is in the form of particles.

A preferred assay format is the sandwich assay. A first antibody capable of binding to fetal human sAChE captures the protein onto a solid surface and a second antibody capable of binding to the protein labels the protein. At least one of the first antibody and the second antibody is a monoclonal antibody of the invention. One of the antibodies, for example the first antibody, may be a polyclonal antibody. The capturing and labelling operations may be performed in any order or simultaneously.

Typically the s cond antibody is labelled with an enzym

such as an alkaline phosphatase or peroxidase. A useful sandwich assay therefore involves contacting a test sample with enzyme-labell d monoclonal antibody, capturing the resulting immune complex onto a solid surface using a monoclonal or polyclonal antibody capable of binding to human sAChE, removing any excess labelled antibody and adding a substrate for the enzyme. The presence of the human sAChE sample is thus revealed.

Other formats which may be employed are any of those suitable for immunoassays including (1) agglutination with the monoclonal antibody adsorbed onto particles of, for example, polystyrene latex; (2) an enzyme-linked immuno-adsorbant assay (ELISA) carried out in microtitre plates, (3) a dipstick ELISA with an antibody-coated dipstick, and (4) sandwich assays using small magnetic particles coated with capture antibody together with the monoclonal antibody labelled either with coloured particles, or particles with the potential of colour development, or with an enzyme or a fluorescent moiety.

Test kits suitable for use in determining human sAChE in a sample comprise a monoclonal antibody according to the invention and means for determining whether the antibody binds to human sAChE. Specific components of the kits may be as described above. The kits may also comprise one or more additional components selected from a control, buffer and diluent.

A kit for use in an enzyme-immunoassay typically includes an enzyme-labelled reagent and a substrate for the enzyme. The enzyme may either be bound to the monoclonal antibody of the invention which can bind to the protein of the invention or be bound to polyclonal or monoclonal antibody capable of binding to the monoclonal antibody.

The following Examples illustrate the invention. A Reference Example is also provided. In the accompanying drawings:

Figure 1 shows the effect of including PEG in the second step of a standard ELISA. Microwell plates coated with fetal sAChE (FsAChE) (200 ng/ml, 100μl), were incubated with dilutions of anti-sAChE culture supernatants followed by incubation with HRO-labelled goat anti-mouse IgM conjugate in the presence (solid line) or absence (broken line) of 4% (w/v) PEG 6000. Colour was developed using tetramethyl benzidine as substrate. Measurements were done in triplicate with standard deviation at <10%. Monoclonal culture supernatants were: AJ2 (●), AJ3 (■) and AJ5 (▲).

Figure 2 shows the results of competitive ELISA (CELIA) using MAB AJ2. The ability of a variety of antigens to inhibit MAB AJ2 binding to immobilised fetal sAChE was tested. AChE activity, as determined by the Ellman assay, of the antigens were as follows: CSF-120 m units/ml; fetal brain DS-AChE-820 m units/ml; PsChE-224 m units/ml; and NHS-53 m units/ml. AJ2 was used at a dilution of 1:250. Assays were done in triplicate and the standard deviation was not greater than 8%. Microtitre wells coated with FsAChE (200 ng/ml, 100ml) were incubated with MAB AJ2 culture supernatant (1:250 dilution) in the presence or absence of dilutions of competing samples. This was followed with HRO-labelled goat anti-mouse Ig conjugate, then, the substrate TMB. Results were the average of triplicate measurements and are expressed as

$$\frac{\text{O.D. in the presence of sample}}{\text{O.D. in the presence of buffer}} \times 100$$

Samples were (*) RBC-AChE, DS-AChE, PsChE and NHS.

(■) AsAChE

(●) FsAChE

(▲) CSF

x-axis AChE concentration scale refers to FsAChE, AsAChE. RBC AChE and PsChE, while the dilution scale refers to CSF, NHS and Ds-AChE.

Figure 3 shows the results of CELIA using MAB AJ5 at a

dilution of 1:25. Experimental details are as for Figure 2.

Figure 4 shows the results of CELIA using MAB AJ3 at a dilution of 1:50. Experimental details are as for Figure 2.

Figure 5 shows the results of an antibody capture sandwich ELISA for sAChE which uses MAB AJ2. Comparison of the binding of different antigens in the sandwich ELISA is shown. Activities of AChE (m units/ml) in the antigen preparations were: fetal brain homogenate-42.6; positive amniotic fluid-62.2; and CSF-120. Microwell titre plates were coated with goat anti-mouse $\mu$ chain antibodies (5$\mu$g/ml, 100 $\mu$l) and then incubated with MAB AJ2 culture supernantant for 5h at room temperature. After washing, dilutions of samples were added and incubation continued for 18h at room temperature. Next, rabbit anti-AChE antiserum was added (2h.room temperature) followed by HRO-labelled goat anti-rabbit IgG conjugate, and substrate TMB. O.D. was read at 450 nm. Samples were, fetal brain homogenate (■), (+) amniotic fluid (●), and cerebrospinal fluid (▲). All assays were done in triplicate with S.D. <10%.

Figure 6 shows the results of an antibody capture sandwich ELISA for sAChE using MAB AJ2. Comparison of binding of maternal serum from pregnancies from a non-NTD fetus (-) and pregnancies from a NTD fetus (+) is shown. Assays were done in triplicate and the standard deviation was <10%. Experimental details are as for Fig 5, but using the following samples:

(+) maternal sera A (■), B (●).

(-) maternal sera F (□), G (○).

REFERENCE EXAMPLE: Isolation and Partial Characterisation of Human Adult and Fetal sAChE

1. EXPERIMENTAL PROCEDURES

Materials

All chemicals were of analytical grade and purchased from Sigma Co., Poole, U.K.

Biological material

Cerebrospinal fluid (CSF) and human adult brain were obtained from the Department of Clinical Chemistry, Southmead Hospital, Bristol, U.K. Human fetal brains were dissected from 12-18 week old aborted fetuses. In both cases tissues were frozen at -70°C within 1h of dissection.

Preparation of tissue homogenate

Extraction of soluble AChE was carried out by thawing the brain tissue and homogenising in 10 volumes (adult brain) or 5 volumes (fetal brain) per weight of ice-cold 0.3M sucrose containing 2 mM EDTA, in a Waring blender (5 x 30s) at 4°C. The homogenate was subjected to two cycles of freezing and thawing to disrupt the membranes and then centrifuged at 100,000 x g for 90 min at 4°C. The supernatant was removed, aliquoted and stored at -70°C until required.

Gel filtration of tissue supernatant

Gel filtration was carried out on Sephacryl (Trade Mark) S-200 (Pharmacia, Uppsala, Sweden) at 4°C. Supernatant samples (40 ml) were applied to a 2.5 cm x 100 cm column equilibrated in phosphate buffered saline, pH 7.3 (phosphate buffered saline (PBS), 142 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$) containing 0.02% $NaN_3$. Elution was by upward flow at a rate of 50 ml/h. Fractions (5 ml) wer coll cted and monitored for (i) protein cont nt

by measuring the optical density at 280 nm and (ii) acetylcholinesterase activity by the Ellman assay (see below). The column was calibrated with a Pharmacia High Molecular Weight kit.

Affinity purification of sAChE

sAChE in the gel filtration eluant was purified further by affinity chromatography on a column of edrophonium chloride-epoxy-Sepharose gel (Pharmacia) prepared according to Hodgson and Chubb (J.Neurochem.,41,654-662,1983). Samples (25 ml) were recycled through a 1 cm x 4 cm column equilibrated in PBS, at a flow rate of 15 ml/h for 24h at 4°C. Unbound material was eluted with PBS containing 0.5 M NaCl (40 ml). The bound material was then eluted with the same buffer containing 12 mM edrophonium chloride (25 ml). Edrophonium chloride was removed by gel filtration on a Sephadex (Trade Mark) -G50 fine (Pharmacia) column (2.5 cm x 50 cm) equilibrated in ammonium acetate (50 mM). Fractions containing AChE activity as determined by the Ellman assay were pooled, lyophilised, reconstituted in PBS (2 ml) and aliquots frozen at -20°C until required.

Enzyme assays

Cholinesterase activity was measured at 30°C by the method of Ellman et al. (Biochem.Pharm.,7,88-95,1961). Enzyme activity is expressed as IU (micromoles product formed per minute). Acetylthiocholine iodide (1 mM) was used as substrate. AChE activity was taken as that activity which was inhibited by 1,5-bis-(4-allyldimethyl-ammonium phenyl)-pentane-3-one dibromide (BW 284 C51; 1.5 μM), while pseudocholinesterase (PsChE) activity was estimated as the difference between two reaction mixtures, one containing eserine (20 μM), the other BW 284 C51 (Hodgson and Chubb, 1983).

Gel electrophoresis analysis of AChE activity

AChE activity in different samples was located on 6% polyacrylamide slab gels (8 cm x 8 cm) by the histochemical method of Coupland and Holmes (Q.J.Microsc.Sci.,98,327-330, 1957), using acetylthiocholine iodide (4 mM) as substrate. The specificity of AChE activity was established by inhibition with BW 284 C51 ($1.5 \times 10^{-4}$M).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially according to Laemmli (Nature,227,680-685,1970) with 8% polyacrylamide slab gels (16 cm x 18 cm). Protein was stained with silver (Nielsen and Brown,Anal.Biochem.,141,311-315,1984). The molecular weight reference standards used were: β-galactosidase (120,000), phosphorylase b (97,000), human IgM μ chain (74,000), bovine serum albumin (65,000), human IgG chain (50,000), aldolase (40,000), carbonic anhydrase (29,000) and human light chain (23,000).

Sucrose density gradient centrifugation

The sedimentation coefficient of sAChE (300 μl) was estimated in 4-20% (w/v) sucrose gradients (5 ml) in 50 mM sodium phosphate buffer, pH 8.0, using β-galactosidase (15.9 S), catalase (11.3 S) and glucose oxidase (7.9 S), as sedimentation markers. Centrifugation was performed at 4°C in a Beckman L5-50 ultracentrifuge with a SW50 rotor (15h at 150,000 xg). Fractions (250 μl) were recovered from the gradient and assayed for AChE activity by the Ellman assay.

Isoelectric focusing (IEF)

Analytical isoelectric focusing was carried out at 10°C on 5% polyacrylamide - 0.5% agarose cylindrical gels (0.4 cm x 8 cm) as described by Wrigley (Meth.Enzymol.,22, 559-564,1971). Ampholytes us d were LKB, pH range 3 - 10

and 4 - 6. Following electrofocusing, gels with sAChE samples were histochemically stained for enzyme activity as described above. The pI of the enzyme was determined from a standard plot constructed by electrofocusing a number of marker proteins of known pI (IEF calibration kit, Sigma Co.)

Lectin Binding

The interaction of sAChE with lectins was investigated by affinity chromatography on Agarose-bound lectins. The following resins were used (Sigma Co.):Agarose-Concanavalin A type III-A, 10 mg lectin/ml gel; Agarose-Lens culinaris 2.5 mg lectin/ml gel and Agarose-ricinus communis, 2.5 mg lectin/ml gel. The gel (100 µl) was washed by centrifugation (three times, 1 ml each) with 50 mM sodium acetate buffer, pH 7.0, containing 0.3M NaCl and 2.5 mM of each of the following salts, $CaCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$. sAChE (10 m unit) was added in the same buffer (400 µl) and the suspension was incubated for 4h at 4°C with gentle mixing. The gel was washed by centrifugation (three times, 0.5 ml each), and the enzyme activity in the unbound material was measured by the Ellman assay. Non-specific binding was determined by using Agarose-bound goat immunoglobulin G gel (Sigma Co.).

Protein Estimation

Protein concentration was determined by using a modified Lowry assay (Markwell et al., Anal.Biochem.,87,206-210, 1978). Bovine serum albumin was used as a standard.

## 2. RESULTS

Extraction of soluble AChE

20 - 30% of the total AChE activity in adult and fetal brains was recovered in the high speed supernatant. Enzyme activities and prot in contents are shown in Table 1.

Table 1 AChE and protein content of human brain high-speed supernatant

| Supernatant | Protein conc. mg/ml | AChE activity Unit/l | AChE activity Unit/mg | ChE Unit/l | Specific activity Unit/mg |
|---|---|---|---|---|---|
| Adult brain | 1.4 | 43.0 | 0.031 | 11.0 | 0.008 |
| Fetal brain | 2.1 | 42.6 | 0.020 | 34.40 | 0.016 |

AChE and ChE activities were measured by the Ellman assay using acetylthiocholine iodine (1 mm) as a substrate and BW 284 C51 (1.5 $\mu$m) as AChE inhibitor. Protein concentration was estimated by Lowry. Protein concentrations and enzyme activity varied by <20% between different preparations.

Analysis of the enzyme activity by PAGE showed that both supernatants contained three bands of esterase activity: a slow moving PsChE band which was not inhibited by BW 284 C51, an intermediate and a fast moving AChE band, both of which were inhibited by BW 284 C51.

Gel Filtration

Brain supernatant was fractionated by gel filtration on a Sephacryl S-200 column. Three major protein peaks were obtained, eluting at molecular weights, $\geq$250,000 (void volume), 50,000 - 60,000 and <10,000, respectively. AChE activity in both supernatants was fractionated into two well-resolved peaks, P1 and P2, with elution volumes corresponding to approximate molecular weights of 220,000-240,000 and 45,000-55,000 daltons, respectively. Fractions corresponding to each peak were pooled. In the adult brain supernatant, the applied AChE activity was qually divided

into the two peaks, while >70% of the fetal supernatant AChE activity appeared in peak P1. Analysis of esterase activity by PAGE showed that P1 contained most of the slow moving AChE (which corresponds to sAChE in CSF) as well as PsChE and some of the fast moving AChE. P2 contained some sAChE, a negligible amount of PsChE and most of the fast moving AChE.

Affinity chromatography of AChE

Adult and fetal P1 and P2 were subjected to affinity chromatography on edrophonium-Sepharose gels. AChE activity in the applied samples, unbound fraction and the edrophonium chloride eluted fraction was determined by the Ellman assay. Between 60-80% of the AChE activity in P1 bound to the affinity column, 40-50% of which could be subsequently eluted with edrophonium chloride, while less than 3% AChE activity in P2 bound. Analysis of the edrophonium chloride-eluted fraction by PAGE showed the presence of one band of enzyme activity which was completely inhibited by BW 284 C51 and had an electrophoretic mobility corresponding to that of sAChE present in CSF. Protein staining of PAGE showed the presence of one band in the edrophonium chloride fraction corresponding to the position of the AChE band. The edrophonium chloride fraction will thereafter be termed sAChE. The specific activity of the purified enzyme following desalting on the Sephadex G50 column was 500-800 units/mg, and 900-1500 units/mg for adult and fetal sAChE, respectively. However, both preparations lost activity rapidly. Hence, following freeze-drying and reconstitution in PBS, the specific activity decreased to 50-100 units/mg. The drop in activity continued even on storage at -20°C. A similar inactivation of the enzyme upon purification was reported for fetal bovine serum sAChE (Chubb et al., Neuroscience, 10(4),1369-1377,1983), and brain AChE (Gordon et al.,

Biochem.J.,157,69-76,1976).

SDS-PAGE

Analysis of adult and fetal sAChE by SDS-PAGE under reducing conditions showed the presence of a predominant band at $M_r$ 66,000. There was no difference in molecular weight between the adult and fetal enzymes.

Sucrose-density gradient centrifugation

Adult sAChE revealed two major forms sedimenting at 15.3 $S_{4W}$ and 10.4 $S_{4W}$. The former is probably aggregated material. A minor peak with a sedimentation constant of 6.25 $S_{4W}$ is also apparent. Fetal sAChE also revealed three forms of AChE activity, though with different relative distribution, at 15.0 $S_{4W}$, 10.5 $S_{4W}$ and 6.1 $S_{4W}$. The 6.1-6.2 S form in both cases increased on storage, even at -20°C. The differences in sedimentation coefficients between the adult and fetal forms of AChE were not significant.

Isoelectric focusing of sAChE

Electrofocusing of purified sAChE was performed using ampholytes with pH ranges of 3-10 and 4-6. Enzyme staining gels revealed one band of activity which was completely inhibited by BW 284 C51. The pI values obtained (n = 10, mean ± S.D.) were: adult sAChE 5.47 ± 0.22, and fetal sAChE 5.47 ± 0.17.

Lectin binding

Adult or fetal sAChE (10m units of each) were mixed with the lectin agarose for 4h at 4°C. After washing, the amount of unbound enzyme in the eluant was determined by the Ellman assay. The results are shown in Table 2. Values are the average of five experiments with th range in brack ts.

Table 2 Binding of sAChE to Lectin columns

| | | % sAChE bound | |
|---|---|---|---|
| Lectin | specificity | adult sAChE | fetal sAChE |
| Con-A | mannose and glucose | 92 (85-98) | 95 (84-97) |
| Lentil | mannose and glucose | 97 (83-99) | 98 (95-99) |
| Ricinus | galactose | 82 (75-89) | 88 (80-90) |
| IgG | nil | 6 ( 3-8 ) | 5 ( 3-9 ) |

Table 2 shows that over 80% of both adult and fetal sAChE was absorbed onto the lectin resins used. This interaction was specific as shown by the lack of binding to Agarose - IgG resin. The binding of sAChE to the lectins was very strong and no more than 50% of the bound enzyme could be desorbed with the corresponding ligand (0.5M α-methyl glucoside, 0.5M α-methyl mannoside and 0.25M β-methyl galactoside).

EXAMPLE 1

1. Methods:

Purification of human fetal and adult sAChE: described in the Reference Example.

Production of polyclonal antisera:

Rabbits were immunised by intramuscular injection with adult sAChE (50 μg) in Freund's Complete Adjuvant (FCA). After 4-5 weeks, the rabbits were injected similarly and bled 5 days later. Antiserum was aliquoted and stored at -20°C.

Production of monoclonal antisera:

Three fusions were performed using fetal sAChE:

(a) In Vivo immunisation: A Balb/c mouse was immunised

intraperitoneally with fetal sAChE (50 μg) in FCA. After 4 weeks, the mouse was injected intravenously with 25 μg of the antigen and fusion with the myeloma cells (X63/Ag8.653) was performed four days later essentially as described by Fazekes and Scheidegger (J.Immunol.Meth.,35,1,1980).

(b) In Vitro immunisation: Spleen cells from an unprimed Balb/c mouse were incubated at a cell density of 1,000,000 cells/ml of medium containing fetal sAChE (50 ng) per ml. The culture medium used was a 50:50 mixture of normal culture medium (10% FCS in RPMI1640 supplemented with glutamine (2 mM) and 2-mercaptoethanol (50 μM)), and thymocyte-conditioned medium (see below). After 5 days in culture the splenocytes were harvested and fused with X63 myeloma cells as described before.

Thymocyte-conditioned medium was prepared as follows; Thymus glands were dissected out of five 12-14 days old Balb/c mice. A single cell suspension was made and the cells were incubated for 3 days at 37°C at a cell density of 5,000,000 cells/ml in 20% FCS in RPMI1640 medium containing glutamine (2 mM) and 2-mercaptoethanol (50 μM). After 3 days the cells were centrifuged and the supernatant (thymocyte-conditioned medium) was filtered (0.22 μM) and stored frozen at -70°C.

(c) In Vivo + In Vitro immunisation: A Balb/c mouse was immunised with fetal sAChE as in (a). After 2 weeks, the spleen was removed and cells subjected to In Vitro immunisation as described in (b).

Screening of hybridomas:

Culture supernatants were initially screened using an ELISA. Microtitre wells were coated with fetal sAChE (200 ng/ml) in 50 mM sodium carbonate/bicarbonate buffer, pH 9.5 overnight at 4°C. After washing with assay buffer (50 mM Tris-HCl, 0.15 M NaCl, 1.0% casein, 0.05% Tween-20, pH 7.2), 100 μl of culture supernatant was added to each

well followed with 100 µl of assay buffer. The plate was incubated overnight at 4°C. Following washes (3x) with assay buffer, 100 µl of horse-radish peroxidas lab 11 d goat anti-mouse immunoglobulin antiserum appropriately diluted in assay buffer were added per well. After 1h incubation at room temperature, the plate was washed and substrate added (tetramethylbenzidine). Colour intensity was measured after 30 min at O.D.450.

Modified ELISA: the assay was performed as above but with the addition of polyethyleneglygol (PEG6000) at 4% (w/vol.) to the second antibody step.

Determination of monoclonal antibody (MAB) specificity:

MAB to sAChE were characterised by using the following methods:

(a) Nitrocellulose Immunodot Binding: Different putative antigen solutions (2 µl) were spotted onto nitrocellulose paper. After drying and blocking with assay buffer, culture supernatants in assay buffer were added and incubation with shaking was performed at room temperature for 4h. The paper was washed with assay buffer and then incubated with goat anti-mouse immunoglobulin-horse radish peroxidase (HRO) conjugate for 2h at room temperature. The paper was washed as before and incubated with substrate solution (3-amino-9-ethyl carbazole) for 30 min.

(b) Competitive ELISA (CELIA): This was performed as the modified ELISA but with one change. The culture supernatant at the appropriate dilution were pre-incubated overnight at 4°C with the unknown prior to addition to the microtitre wells.

(c) Immunoaffinity Chromatography: The MAB was covalently bound to solid phase as follows: Culture supernatant (50 ml) was mixed with Sepharose-4B-goat anti-mouse IgM antibody gel (1 ml) (Sigma Co.) for 2h at room temperature followed by overnight incubation at 4°C.

Unbound material was washed away and the IgM-anti-IgM complex was cross-linked as follows: the gel-antibody complex was incubated with 0.2 mg/ml dimethyl suberimidate in 0.2M triethanolamine buffer, pH 8.4 for 3h at room temperature to covalently cross-link the MAB to the anti-IgM on the Sepharose gel. The reaction was terminated by the addition of 0.5M lysine. The gel was washed with PBS and stored in the same buffer containing 0.2% sodium azide.

Immunoaffinity absorption of AChE: Sepharose-MAB gel (0.2 ml) was mixed with various antigen solutions made up to 500 µl in PBS. Incubation was carried out for 2h at room temperature, then overnight at 4°C. Triton X-100 (0.1% (w/v)) was added to the detergent-soluble antigens. After incubation, the gel and any bound antigen were spun down at low speed and the gel was washed twice with PBS. All unbound material was kept for analysis by the Ellman enzyme assay and by PAGE.

Enzyme assay for AChE:

ChE and AChE activity was assayed by the Ellman test.

PAGE analysis of ChE and AChE:

Polyacrylamide gel electrophoresis was performed as described by Coupland and Holmes (Q.J. Microsc.Sci.,98, 327-330).

Sandwich ELISA for AChE:

Microtitre wells were coated overnight with goat anti-mouse IgM antibodies (Sigma) (5 µg/ml, 100 µl) overnight at 4°C. After washing and blocking with assay buffer, 100 µl of dilutions of culture supernatant was added and the plate incubated for 5h at room temperature. After washing, samples containing antigens were added and incubation carried out overnight at room temperature. The plate was washed and an appropriat dilution of rabbit anti-AChE

antiserum in assay buffer was added. After 2h incubation, HRO-labelled goat anti-rabbit IgG was added follow d by the substrate. In an alternative assay, following the incubation with the antigen solution, biotin-labelled rabbit anti-AChE immunoglobulin fraction was added at the appropriate dilution for 2h, followed by horse-radish peroxidase labelled streptavidin (Sigma), then by the substrate.

2. RESULTS

Purification of AChE: described in the Reference Example. In summary, purified fetal and adult sAChE were obtained from human brain. Gel filtration, sucrose-density gradient centrifugation and SDS-PAGE analyses indicate that the enzyme is a tetramer with a molecular weight of around 240,000 (10.15 S) consisting of four subunits of molecular weight of 66,000. The enzyme has a pI of 5.47.

Polyclonal anti-sAChE antisera:

Four rabbit antisera were raised to the adult form of sAChE. Analysis by CELIA showed them all to cross react with other AChE forms. These antisera were used later on in setting up sandwich ELISAs.

Monoclonal anti-sAChE antibodies:

Both fusion experiments (a) and (b) (see Methods section) produced more than 90% of wells positive for hybridomas, but no anti-AChE antibodies were detected. Hence, it was decided to use a combination of In Vitro and In Vivo immunisations (fusion experiment (c)). The modified ELISA was used as a screening assay. Addition of PEG to the MAB incubation step, i.e. to the step of incubating a culture supernatant with fetal sAChE-coated microtitre wells, actually reduced the sensitivity f the

ELISA to detecting MABs specific for fetal sAChE. Howev r, PEG addition to th second antibody incubation step at 4% (w/v) greatly enhanced the detection limit.

Figure 1 shows the remarkable effect of PEG on the titration of three MAB to fetal sAChE. This experiment was done using MAB from hybridomas that had been cloned three times and whose antibody content was quite high. Addition of PEG was routinely done to all subsequent assays.

Fusion experiment (c) produced a number of positive hybrids. Nine of these were cloned three times by limiting dilution. Results obtained with some of these are reported here. All the nine MAB were of the IgM class. A test of specificity for six of these MAB was effected by the dot blot method. All six MABs showed a preference of binding to the fetal sAChE to which they were raised.

Characterisation of MABs: by CELIA:

ELISA titration curves of culture supernatants were obtained for the nine MABs. The specificity of these were checked by the more sensitive CELIA. Figures 2, 3 and 4 illustrates the results obtained with MABs AJ2, AJ5 and AJ3, respectively. All three MABs did not cross react with human RBC-AChE, pChE, normal human serum (NHS) and detergent-soluble membrane AChE from human fetal brain. All these are known not to contain sAChE. The strongest reactivity was obtained with purified fetal sAChE and less reactivity with purified adult sAChE, and CSF (which contains only adult sAChE).

Characterisation of MAB by Immunoaffinity Chromatography:

The specificity of MAB to sAChE was further tested by immunoaffinity absorption. The MABs were immobilised and covalently-linked to Sepharose-4B-anti-IgM gel for two r asons: (i) to allow multiple use of the gel and (ii) the covalent cross-linkage was found to stabilis antibody

activity of the MAB.

Results of all the binding studies carried out with MAB AJ2, AJ3 AND AJ5 are shown in Table 3. The Table shows the percentage of enzyme activity in the samples which bound to each MAB affinity column. The amount of activity bound to the edrophonium-Sepharose column is shown for comparison. Table 4 shows the AChE and pChE enzyme activities of the antigen solutions used in the binding studies. These activities were determined by the Ellman assay.

TABLE 3: Immunoaffinity Absorption Chromatography

| Antigen | AJ2 mAb column | AJ3 mAb column | AJ5 mAb column | Edrophonium-Sepharose column |
|---|---|---|---|---|
| Fetal brain homogenate | 67% AChE<br>0 PsChE | 65% AChE<br>0 PsChE | 69% AChE<br>0 PsChE | 61% AChE<br>0 PsChE |
| Human serum PsChE | 0 | 0 | 0 | 0 |
| NHS | 0 | 0 | 0 AChE<br>7% PsChE | 0 |
| Detergent-soluble AChE | 0 | 0 | 9% AChE<br>0 PsChE | 0 |
| Red blood cell AChE | 0 | 0 | 0 | 0 |
| CSF | 48% AChE<br>0 PsChE | not tried | not tried | 85% AChE<br>0 PsChE |

Table 4: Activities of the various antigen solutions

| Antigen | AChE activity (m units/ml) | PsChE activity (m units/ml) |
|---|---|---|
| Adult brain homogenate | 43.0 | 11.0 |
| Fetal brain homogenate | 42.6 | 34.0 |
| CSF | 120.0 | 50.0 |
| Detergent-soluble AChE (fetal) | 820.0 | 48.2 |
| Human serum PsChE | 224.0 | 1970.0 |
| Human red blood cell AChE | 1000.0 | 120.0 |
| NHS | 53.0 | 658.6 |
| Amniotic fluid | 62.2 | 87.5 |

It can be seen from Table 3 that only sAChE is bound to MAB AJ2 and MAB AJ3 columns. RBC AChE, pChE, detergent soluble brain AChE and non-secretory type soluble AChE did not bind to these columns. In fetal brain homogenate, 67% of the AChE activity was bound by the AJ2 column, which compares well with the 61% AChE bound from this material by the edrophonium-Sepharose column. All this bound AChE is presumed to be sAChE, as the edrophonium-Sepharose gel is specific for sAChE. Binding of sAChE in CSF to the AJ2 column was also carried out by the same method and 48% bound as opposed to 85% binding to the Edrophonium-Sepharose gel. This again suggests that Mab AJ2 possibly has greater affinity for sAChE from a fetal, rather than adult, origin.

Immunoassay for sAChE:

A sandwich ELISA was set up using MAB AJ2 and the polyclonal rabbit anti-AsAChE antiserum. This assay was then used with some AF samples and maternal sera obtained from pregnancies with known outcome.

Figure 5 shows titration curves for fetal brain homogenate, CSF and a positive (+) AF (AF from an NTD pregnancy). It can be clearly seen that the assay can detect sAChE in (+) AF. The Figure also indicates that the greatest activity is found in the CSF and the least in the fetal brain homogenate.

This is different from the results obtained with CELIAs, where results indicate greater activity in the fetal brain homogenate than in the CSF. The reason for this, is most likely to do with assay format. In the Sandwich ELISA the detection relies on the use of rabbit anti-AChE antiserum. This, as explained earlier, was raised to the adult sAChE. Therefore the results may reflect greater affinity of this antiserum to the adult rather than the fetal form of sAChE.

This assay using HRO-labelled anti-rabbit IgG as the

revealing system was used with a number of (+) AF and (-) AF samples. Table 5 shows the results obtained.

Measurements were done in triplicate and the standard deviation was <10%. Both (+) and (-) samples showed high absorbance. However, the average absorbance given by the (+) AF samples was markedly higher than that of the (-) AF samples.

The same assay was applied to a (+) and (-) maternal sera. Figure 6 shows the titrations obtained with two samples of each. Once again both gave high absorbance readings.

Improvement of the assay was attempted using a biotin/ streptavidin revealing system. This gave better results in term of low background and sensitivity. Table 6 shows the results obtained with (+) and (-) AF and maternal sera samples.

Table 5: Antibody capture sandwich ELISA using MAB AJ2

| Amniotic Fluid Sample | Optical density 450nm Dilution of sample | | | |
|---|---|---|---|---|
| | 1:2 | 1:4 | 1:8 | 1:16 |
| 1 (+) | 1.33 | 0.86 | 0.33 | 0.35 |
| 2 (+) | 1.39 | 1.12 | 0.83 | 0.71 |
| 3 (+) | 1.25 | 1.20 | 0.97 | 0.81 |
| 4 (+) | 0.92 | 0.59 | 0.24 | 0.26 |
| 5 (+) | 0.80 | 0.79 | 1.04 | 0.24 |
| 6 (+) | 1.23 | 0.88 | 0.66 | 0.73 |
| Mean (+) | 1.15 | 0.91 | 0.68 | 0.52 |
| 7 (-) | 1.16 | 0.86 | 0.84 | 0.21 |
| 8 (-) | 1.11 | 1.00 | 0.40 | 0.28 |
| 9 (-) | 1.19 | 0.89 | 0.48 | 0.12 |
| 10 (-) | 0.78 | 0.65 | 0.43 | 0.18 |
| 11 (-) | 1.05 | 1.13 | 0.52 | 0.16 |
| Mean (-) | 1.06 | 0.91 | 0.53 | 0.19 |

Table 6

| Sample | Optical density | | | Average O.D. 450nm | |
|---|---|---|---|---|---|
| 1 AF (+) | 0.83 | 0.81 | 0.84 | 0.83 | |
| 2 AF (+) | 0.64 | 0.84 | 0.84 | 0.77 | 0.76 |
| 3 AF (+) | 0.68 | 0.77 | 0.59 | 0.68 | |
| 8 AF (-) | 0.39 | 0.73 | 0.73 | 0.62 | |
| 10 AF (-) | 0.65 | 0.54 | 0.47 | 0.55 | 0.61 |
| 11 AF (-) | 0.68 | 0.60 | 0.66 | 0.65 | |
| C S (+) | 1.57 | 1.30 | 1.21 | 1.36 | |
| A S (+) | 1.94 | 1.79 | 1.53 | 1.75 | 1.42 |
| D S (+) | 1.20 | 1.11 | 1.10 | 1.14 | |
| F S (+) | 1.80 | 1.45 | 1.51 | 1.42 | |
| G S (-) | 0.67 | 1.02 | 0.85 | 0.85 | |
| F S (-) | 0.58 | 0.80 | 0.91 | 0.76 | 0.76 |
| H S (-) | 0.71 | 0.81 | 0.67 | 0.73 | |
| J S (-) | 0.71 | 0.76 | 0.61 | 0.69 | |

Blank value (n = 3) 0.64

AF+ amniotic fluid positive for sAChE

AF- amniotic fluid negative for sAChE

S+ serum from a known NTD pregnancy

S- serum from a known non-NTD pregnancy

EXAMPLE 2

Materials

All chemicals were of analytical grade and purchased from Sigma Co, Poole, U.K. Biological materials were obtained as described in the Reference Example.

Preparation of tissue homogenate

Extraction of fetal soluble AChE was carried out by thawing the brain tissue and homogenising in 5 volumes (fetal brain) per weight of phosphate buffered saline (PBS) containing 2 mM EDTA, in a Waring blender (5 x 30s) at room temperature. The homogenate was subjected to two cycles of freezing and thawing to disrupt the membranes, diluted 4-fold with PBS containing 2mM EDTA and then centrifuged at 18,000 x g for 90 min at 4°C. The supernatant was removed, aliquoted and stored at -70°C until required.

Affinity purification of sAChE

sAChE was purified from the fetal brain homogenate by direct affinity chromatography on a column of edrophonium chloride-epoxy-Sepharose gel (Pharmacia) prepared according to Hodgson and Chubb (J. Neurochem., 41, 654-662, 1983). Up to 500 ml of fetal brain homogenate were recycled through a 1 cm x 7 cm column equilibrated in PBS, at a flow rate of 1 ml/min for 24 h at room temperature. Unbound material was eluted with PBS containing 0.5 M NaCl (35 ml). The bound material was then eluted with the same buffer containing 12 mM edrophonium chloride (35 ml). Edrophonium chloride was removed by gel filtration on a Sephadex (Trade Mark) -G50 fine (Pharmacia) column (2.5 cm x 50 cm) equilibrated in ammonium acetate (50 mM). Fractions containing AChE activity as determined by the Ellman assay were pooled, lyophilised, reconstituted in PBS (2 ml) and aliquots frozen at -20°C until required.

This preparation gave pure fsAChE as determin d by SDS-PAGE, visualised by silver staining. Fetal calf s rum AChE

(FCS AChE) was prepared in the same way, except the fetal calf serum (Gibco, U.K.) was recycled through the column up to 100 ml at a time.

Production of FCS AChE, or fetal secretary, AChE (human) carrier protein conjugates

20 μg of AChE was coupled to 200 μg of keyhole limpet hemocyanin (KLH) (Pierce Co, England) by incubation with 0.5 ml of 0.1% glutaraldehyde in PBS at room temperature for 16 hr. The produced was dialysed versus PBS to remove any unreacted glutaraldehyde.

Production of monoclonal antibody hybridomas)

Eight fusions were performed using fetal sAChE:

(A) Fusion 1: Immunisation with fetal sAChE coupled to KLH.

An F1 hybrid mouse (CFLP x Balb/c cross) was immunised by subcutaneous inoculation with fetal sAChE/KLH conjugate (60μg) in Freundo Complete Adjuvant. After 8 days the mouse was boosted by inoculation into the peritoneal cavity of 60μg of fetal sAChE/KLH conjugate in Freunds Incomplete Adjuvant. After 6 days 5 μl of blood was taken by superficial venesection tail and assayed by direct ELISA for reactivity of antibodies with fetal sAChE as coating antigen. The antisera proved positive even when diluted down by $1/10^5$. Four days later a further inoculation of 60μg of fetal sAChE/KLH conjugate was made into the peritoneal cavity in PBS buffer. 7 days later a final booster inoculation into the peritoneal cavity was made of 30 μg fetal sAChE/KLH conjugate in PBS. After 4 days splenocytes were removed and fused with myeloma cells (x63/Ag8.653) essentially as described by Fazekas and Scheidegger (J. Immunol. Meth., 35, 1, 1980).

(B) Fusions 2-4 and 7-8 Intrasplenic immunisation:

Thr e F1 hybrid mice (CFLP x Balb/c cross) - Fusions

2-4 and two Balb/c mic - Fusions 7 and 8 were anaesthetised with hypnorm/midazolam. The fur was clipped over the spleen and an incision 1-1.15 cm long was made just below the left set of ribs. The muscle was separated by blunt dissection, and the lower pole of the spleen was exteriorised through a small incision made in the peritoneum. Fetal sAChE 9$\mu$g in 50$\mu$l PBS was inoculated directly into the spleen via a 27g x 27cm (1/2 inch) needle. The spleen was pushed back into the peritoneal cavity, and incision was closed using metal stitch clips. Splenocytes were removed after 4 days and fused with myeloma cells (x63/Ag8.653) essentially as described by Fazekas and Scheidegger (J. Immunol. Meth, 35, 1, 90).

(c) Fusions 5 and 6: In vitro immunisation

(i) Thymus conditioned medium was prepared by forming a mixed thymocyte culture of thymocyte from 6 three week old Balb/c mice and from 6 three week old CFLP mice. They were cultured for 24 hrs or 48 hrs in Dulbeccos Modified Eagles Medium (DMEM) with 5% (v/v) Myoclone fetal calf serum + 5 x $10^{-5}$M 2-mercaptoethanol + HT supplement (Sigma) + 18mM HEPES pH 7.3. Cells were cultured at a density of 5 x $10^6$/ml in 25 $cm^2$ flasks. The supernatant was harvested, filtered (0.22$\mu$m) and stored at -20°C.

(ii) EL4 supplement.

EL4 cells (obtained from ECACC, Porton Down) were cultured at 1 x $10^6$ cells/ml in DMEM at 5% myoclone FC5 + 5 x $10^{-5}$M 2-mercaptoethanol + HT supplement + 18mM HEPES pH 7.3 + 10ng phorbol myristate acetate/ml for 40 hrs. The supernatant was harvested, filtered (0.22 $\mu$m) and stored at -20°C.

In vitro culture

Splenocytes were removed from an F1 mouse (Balb/c x CFLP

cross) and cultured at 5 x $10^6$ cells/ml in medium containing 0.8 µg/ml fetal sAChE. Th medium used was Dulbeccos Modified Eagles Medium + 5% v/v myoclone fetal calf serum + 33% (v/v) thymus conditioned medium + 25% (v/v) EL4 supoplement + 5 x $10^{-5}$M 2-mercaptoethanol + HT supplement + 18 mM HEPES pH 7.3. Two 25$cm^2$ flasks were set up. After 6 days stimulated cells from one flask were fused with myeloma cells (x63/Ag8.653) essentially as described by Fazekas and Scheidegger (J. Immunol. Meth. 35, 1, 90). This was fusion 6.

The cells in the second flask were restimulated for a further 6 days with 0.8µg/ml fetal sAChE in fresh medium prior to fusion with myeloma cells by the same method. This was fusion 5.

Screening of Hybridomas

Culture supernatants were initially screened using a direct ELISA. However due to the very low signals leading to spurious results, cells were either pulsed with serum-free medium at least 1 day prior to screening with the original direct ELISA or were grown solely in serum-free medium. This had the effect of making sure none, or very little (<1%) fetal calf serum was present in the supernatant at the time of screening.

Direct ELISA for screening hybridomas

Microtitre wells were coated with human fetal sAChE (200 ng/ml) in 50 mM sodium carbonate/bicarbonate buffer, pH 9.6 overnight at room temperature. Fetal sAChE when available in larger quantity was used at 400 ng/ml. After washing thrice with assay buffer (phosphate buffered saline, pH 7.2, 0.05% Tween-20), 100 µl of culture supernatnat was added to each well and the plate incubated for 2 h at 37°C. Following washes (5x) with assay buffer, 100µl of horse-radish peroxidase labelled goat-anti mouse immunoglobulin

antiserum appropriately diluted in assay buffer was added per well. After 1 hr incubation at 37°C, the plat was washed (5x) with assay buffer and substrate added (tetramethylbenzidine solution). After stopping the reaction with 50µl of 2M $H_2SO_4$, column intensity was measured at O.D. 450.

Determination of monoclonal antibody (MAB) specificity by ELISA

Microtitre wells were coated with human fetal sAChE (400 ng/ml) or Red blood cell AchE (5µg/ml) in 50mM sodium carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C 200µl/well at all stages. After blocking for 30 min, and washing (2x) with assay buffer (50mM Tris/HCl, 0.05% Tween-20, 0.02% sodium azide, pH 7.4), 200µl of each MAB diluted in assay buffer plus 0.1% casein was added to each well and the plate incubated for three hours at room temperature. Following washing (3 x 10 mins) with assay buffer, wells were incubated with an alkaline phosphate conjugated goat anti mouse immunoglobulin conjugate appropriately diluted in assay buffer plus 0.1% casein plus 4% PEG 6000 for one hour at room temperature. Following washing with assay buffer substrate solution (1mg/ml p-nitrophenyl phosphate disodium in 10% w/v diethanolamine-HCl, 0.01% magnesium chloride, 0.02% sodium azide, pH 9.8) was added and incubated for 1 hr at room temperature prior to reading at 405 nm.

## RESULTS

Hybridomas secreting monoclonal antibodies reactive with fetal sAChE after cloning

Assays were performed using the new direct ELISA from serum-free medium supernatants.

Fusion 1: Immunized and boosted *in vivo* with fetal sAChE

coupled to KLH. From an F1 mouse. Two cloned hybridomas were created.
AB 1-1
AB 1-7

Fusion 2: Intrasplenic: F1 mouse. Two cloned hybridomas were created.
AB 2-2
AB 2-3.

Fusion 3: Intrasplenic: F1 mouse. Three cloned hybridomas were created.
AB 3-2
AB 3-5 (deposited at the European Collection of Animal Cell Cultures, Porton Down, GB on 3 December 1990 under accession number 90120322.
AB 3-7.

Fusion 4: Intrasplanic: F1 mouse. One cloned hybridoma was created.
AB 4-3
AB 4-7.

Fusion 5: in vitro immunisation, restimulated: F1 mouse. Seven cloned hybridoms were created..
AB 5-1
AB 5-7
AB 5-8
AB 5-9
AB 5-12
AB 5-14
AB 5-15

Fusion 6: in vitro immunisation, F1 mouse. Five cloned hybridomas were created.

AB 6-2
AB 6-3
AB 6-5
AB 6-7
AB 6-10

Fusion 7: Intrasplenic immunisation Balb/c mouse. Six cloned hybridomas were created:
AB 7-1
AB 7-3
AB 7-6
AB 7-7
AB 7-9
AB 7-10.

Fusion 8: Intrasplenic immunisation, Balb/c mouse. Five cloned hybridomas were created:
AB 8-1
AB 8-2
AB 8-4
AB 8-5
AB 8-6.

The specificity of monoclonal antibodies, as determined by ELISA, is shown in Table 7 below. Results are given as OD 405.

TABLE 7

| Clone name | antibody concentration or dilution | FsAChE as antigen | | | Red Blood Cell AChE as antigen | | |
|---|---|---|---|---|---|---|---|
| | | 1st reading | 2nd reading | | 1st reading | 2nd reading | |
| AB 4-7 | 1.2 mg/ml | 0.611 | 0.618 | +ve | 0.205 | 0.215 | -ve |
| AB 5-12 | 1.8 mg/ml | 1.148 | 1.053 | + | 0.225 | 0.239 | - |
| AB 3-5 | 1.5 mg/ml | 2.337 | 2.202 | + | 0.309 | 0.295 | - |
| AB 6-3 | 0.05 mg/ml | 0.786 | 0.754 | + | 0.211 | 0.213 | - |
| AB 5-15 | Ascites 1/100 | 0.699 | 0.637 | + | 0.358 | 0.350 | - |
| AB 7-1 | Ascites 1/100 | 1.662 | 1.576 | + | 0.463 | 0.501 | weak |
| AB 2-3 | Ascites 1/100 | >3.00 | >3.00 | + | >3.00 | >3.00 | +ve |
| AB 7-3 | Ascites 1/100 | 1.463 | 1.479 | + | 0.310 | 0.324 | -ve |
| AB 8-1 | Ascites 1/100 | 0.586 | 0.603 | + | 0.202 | 0.210 | -ve |
| AB 1-7 | Ascites 1/100 | >3.00 | >3.00 | + | 2.541 | 2.620 | +ve |
| AB 5-9 | Ascites 1/100 | 1.676 | 1.679 | + | 1.448 | 1.463 | +ve |
| AB 8-2 | Ascites 1/100 | 0.703 | 0.708 | + | 0.462 | 0.521 | weak |
| AB 6-10 | Ascites 1/100 | 1.253 | 1.257 | + | 0.613 | 0.601 | |
| AB 7-9 | Ascites 1/100 | 0.644 | 0.689 | + | 0.291 | 0.401 | weak |
| AB 7-10 | Ascites 1/100 | 0.849 | 1.054 | + | 0.293 | 0.364 | -ve |
| AB 7-7 | Ascites 1/100 | 0.595 | 0.541 | + | 0.505 | 0.584 | + |
| AB 1-1 | Ascites 1/100 | | | +ve | | | +ve |

AB 4-7, AB 5-12, AB 3-5 were from ammonium sulphate cuts of crude ascites

AB 6-3 was a euglobulin precipitation from crud ascites.

Cells secreting antibody not tested yet or positive ascites not yet produced include AB 2-2, AB 3-2, AB 3-7, AB 4-3, AB 5-1, AB 5-7, AB 5-8, AB 5-14, AB 6-2, AB 6-5, AB 6-7, AB 7-6, AB 8-4, AB 8-5, AB 5-6.

This experiment shows that specific antibody-secreting cells have been produced from fusion 3 (AB 3-5) fusion 4 (AB 4-7), fusion 5 (AB 5-12), fusion 6 (AB 6-3), fusion 7 (AB 7-3, AB 7-10) and fusion 8 (AB 8-1).

CLAIMS

1. A monoclonal antibody which is specific for human secretory acetylcholinesterase (sAChE) and which shows no cross-reactivity with other types of human acetyl-cholinesterase.

2. A monoclonal antibody according to claim 1, which is an IgM or IgG.

3. An immortalised cell line which secretes a monoclonal antibody as claimed in claim 1 or claim 2.

4. A process for the preparation of a monoclonal antibody as claimed in claim 1 or 2, which process comprises :
(a) culturing an immortalised cell line which secretes the monoclonal antibody; and
(b) isolating the monoclonal antibody thus produced.

5. A process for the preparation of an immortalised cell line as claimed in claim 3, which process comprises :
(I)(a) immunising an animal with adult or fetal human sAChE;
(b) obtaining lymphatic tissue cells from the immunised animal;
(c) culturing the obtained cells with adult or fetal human sAChe;
(d) immortalising the cultured cells; and
(e) screening the resulting cell lines for an immortalised cell line which secretes monoclonal antibody which is specific for human sAChE; or
(II)(a) obtaining lymphatic tissue cells from an aminal,
(b) culturing the obtained cells with adult or fetal human sAChE,
(c) immortalising the cultured cells, and
(d) screening the resulting cell lines for an immortalised cell line which secretes monoclonal antibody which is specific for human sAChE; or
(III)(a) obtaining lymphatic tissue cells from an animal,

(b) culturing the obtained cells with adult or fetal human sAChE,

(c) culturing the cells obtained in step (b) with fresh adult or fetal human sAChE,

(d) immortalising the cells obtained in step (c), and

(e) screening the resulting cell lines for an immortalised cell line which secretes monoclonal antibody which is specific for human sAChE; or

(IV)(a) immunising an animal with adult or fetal human sAChE by inoculation directly into a lymphatic organ or lymph node,

(b) obtaining lymphatic tissue cells from the immunised animal,

(c) immortalising the cells obtained, and

(d) screening the resulting cell lines for an immortalised cell line which secretes monoclonal antibody which is specific for human sAChE.

6. A process according to claim 5, wherein splenocytes are obtained in step (b) of process (I) or (IV) or in step (a) of process (II) or (III) and the cultured cells are fused with myeloma cells in step (d) of process (I) or (III) or in step (c) of process (II) or (IV).

7. A process according to claim 5 or 6, wherein resulting cell lines are screened by

- contacting a solid support coated with adult or fetal human sAChE with supernatant from a culture of the cell line and with, in the presence of polyethylene glycol, a labelled antibody capable of binding to any monoclonal antibody in the supernatant which binds to the human sAChE; or

- contacting a solid support coated with adult or fetal human sAChE with supernatant from a culture of each cell line grown in serum-free or low serum-supplemented medium and with a labelled antibody capable of binding to any monoclonal antibody in the supernatant which binds to th

human sAChE.

8. A method of determining human sAChE in a sampl , which method comprises carrying out a said det rmination using a monoclonal antibody as claimed in claim 1 or 2.

9. A method according to claim 8, comprising contacting a sample suspected of containing human sAChE with a said monoclonal antibody and determining whether the said monoclonal antibody has bound to any human sAChE.

10. A test kit suitable for use in determining fetal human sAChE, which kit comprises a monoclonal antibody as claimed in claim 1 or 2 and means for determining whether the monoclonal antibody has, in use, bound human sAChE.

11. A monoclonal antibody which is specific for human sAChE and which shows no cross-reactivity with human red blood cell AChE.

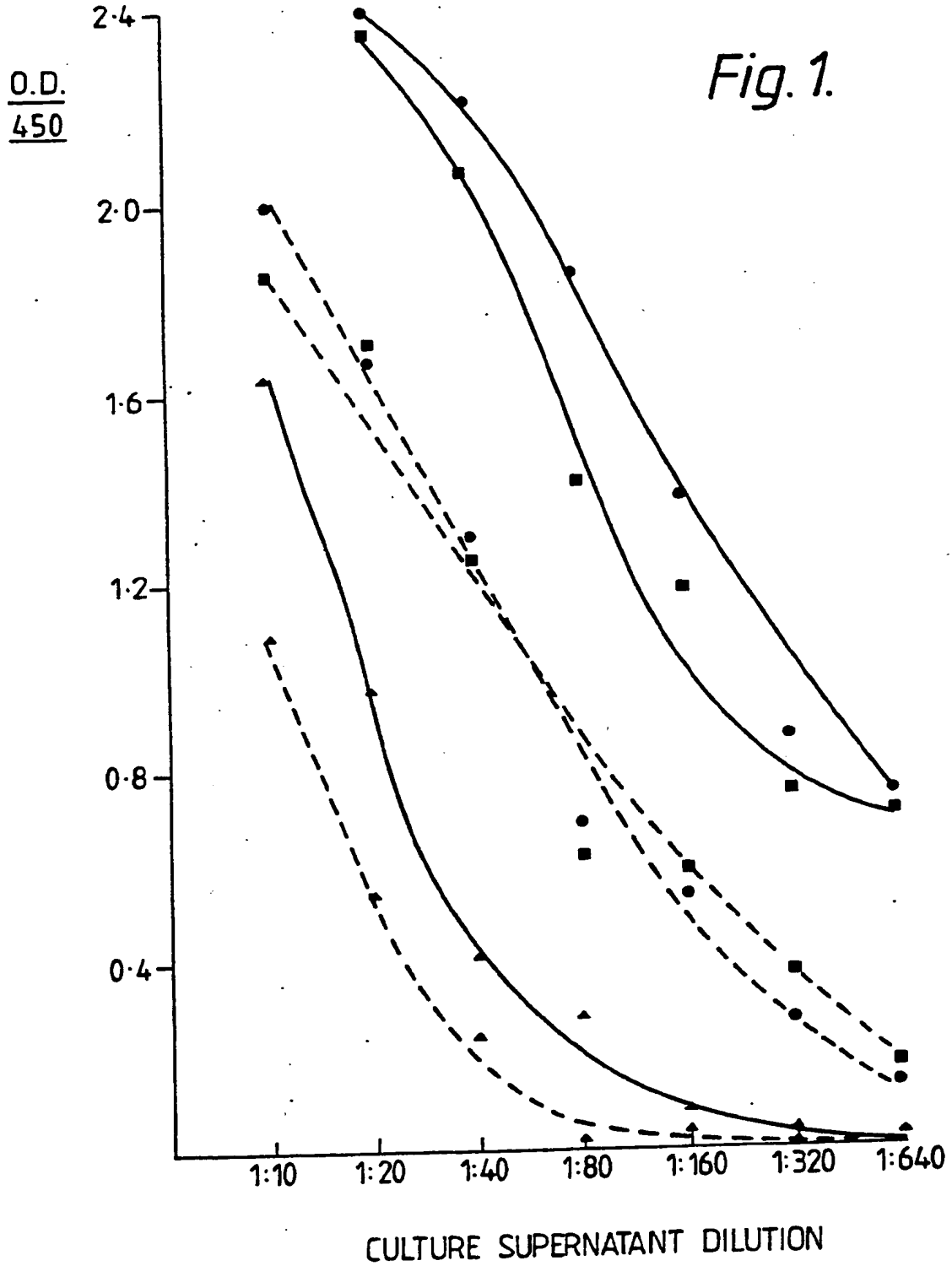


Fig. 1.

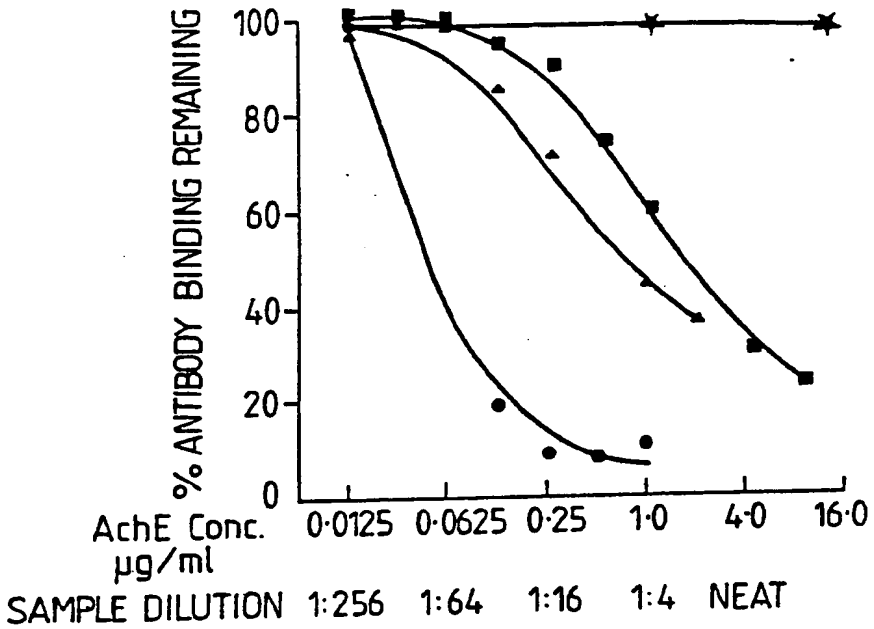


Fig. 2.

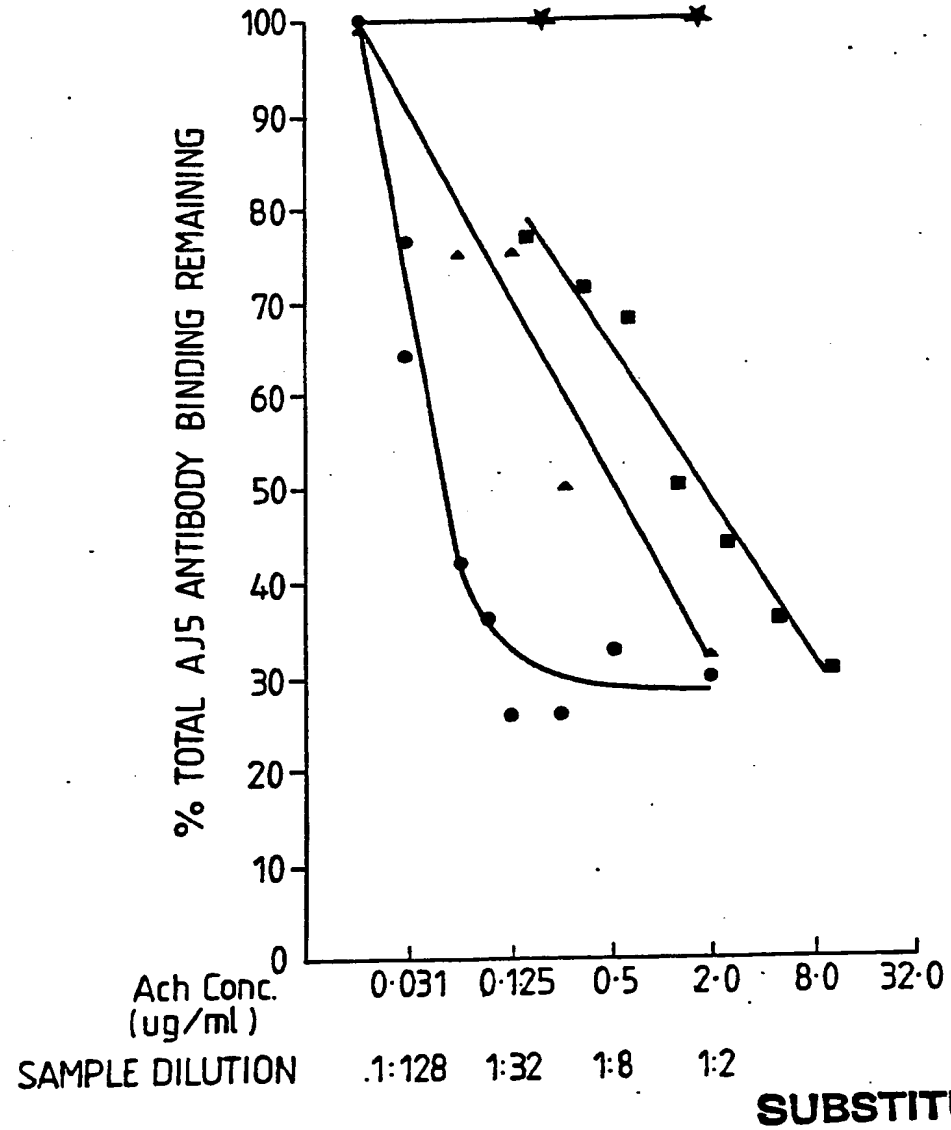


Fig. 3.

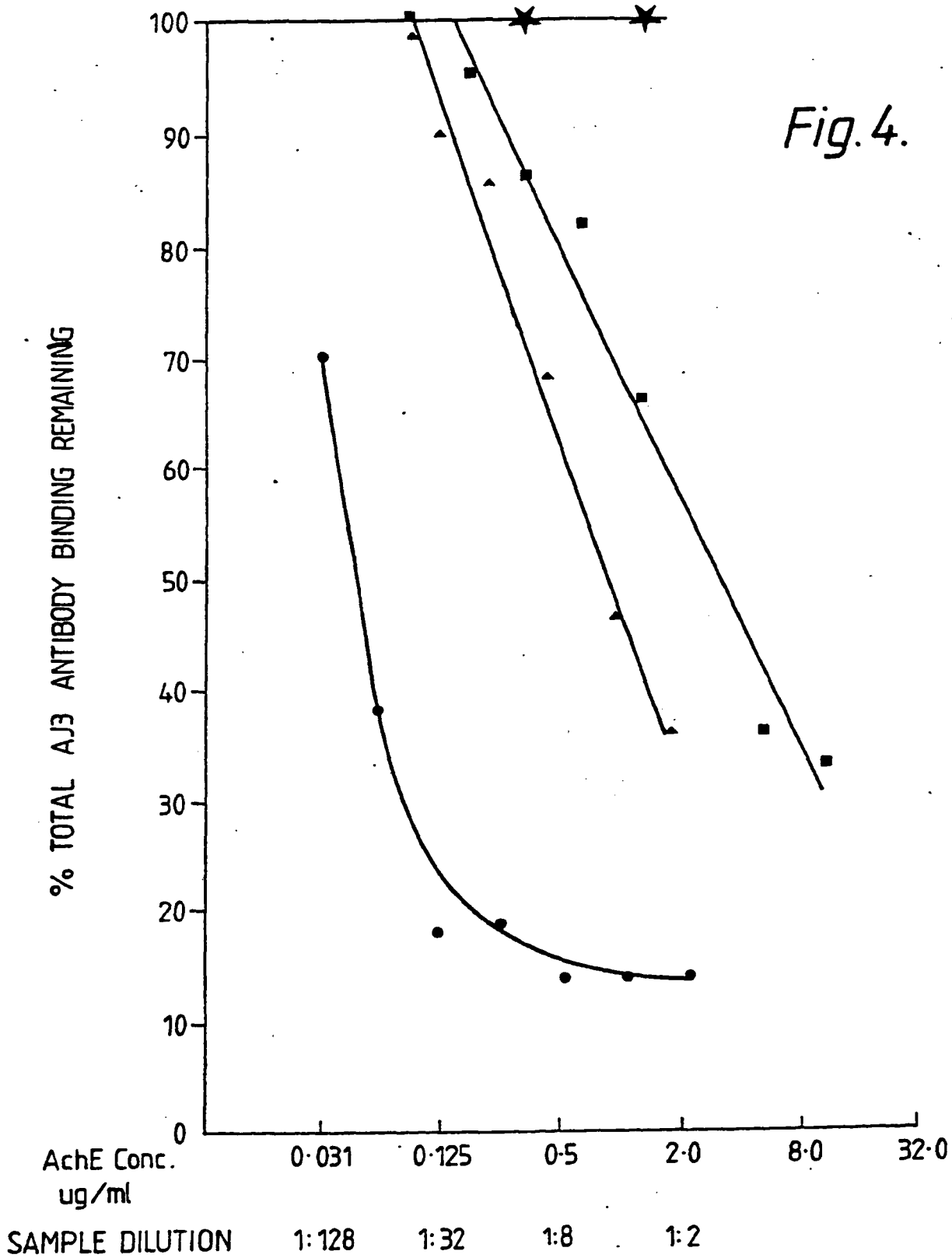


Fig. 4.

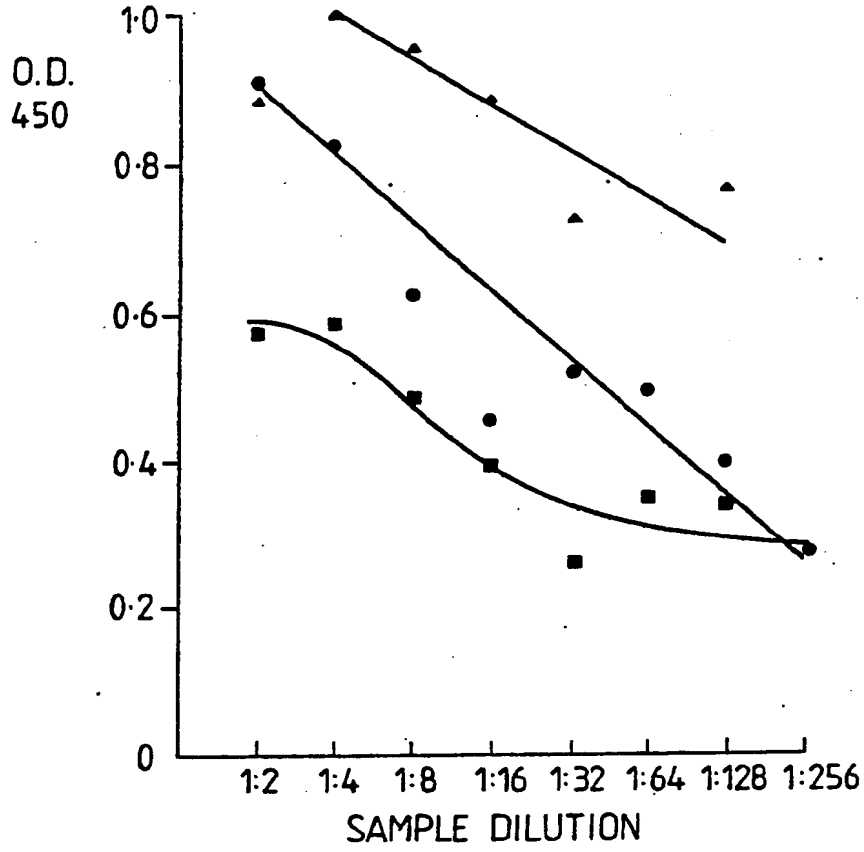


Fig. 5.

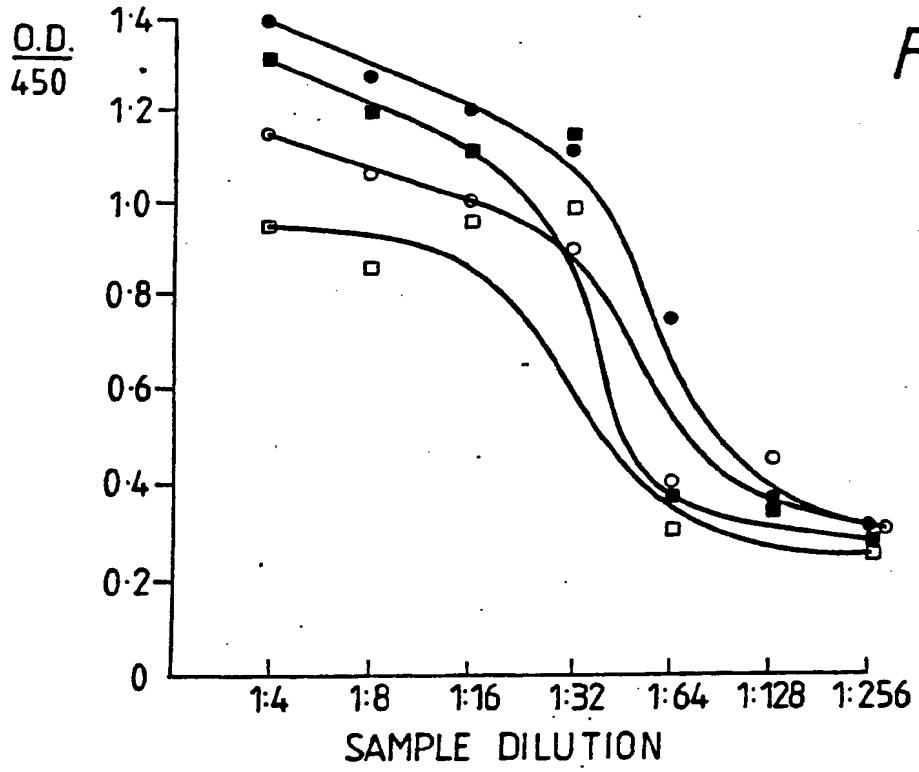


Fig. 6.

# .TERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01886

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12P21/08 ; C12N5/20 ; C12N15/06 ; G01N33/573

## II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | C12P ; C12N ; C07K ; G01N |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category[10] | Citation of Document,[11] with indication, where appropriate, of the relevant passages[12] | Relevant to Claim No.[13] |
|---|---|---|
| X | CLIN. CHIM. ACTA<br>vol. 165, no. 1, June 1987, Amsterdam, NL<br>pages 17 - 25; A. RASMUSSEN et al.:<br>"Immunochemical determination of acetylcholinesterase in amniotic fluid. An evaluation of eleven monoclonal antibodies."<br>see page 21, line 10 - page 23, line 15<br>(cited in the application) | 1-11 |
| Y | BIOCHEM. SOC. TRANS.<br>vol. 14, no. 6, March 1986, London, GB.<br>pages 1234 - 1235; P. GARDNER et al.:<br>"Isolation and partial characterization of the secretory form of human brain acetylcholinesterase."<br>see the whole document<br>(cited in the application) | 1-11 |

-/--

[10] Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 13 FEBRUARY 1991 | 2 MAR 1991 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | NOOIJ F.J.M. |

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category ° | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| Y | NATURE.<br>vol. 256, 1975, LONDON GB<br>pages 495 - 497; G. KÖHLER & C. MILSTEIN:<br>"Continuous cultures of fused cells secreting antibody to predefined specificity."<br>see the whole document<br>--- | 1-6, 11 |
| Y | J. IMMUNOL. METHODS<br>vol. 44, no. 2, 1981, Amsterdam, NL<br>pages 125 - 133; S. COBBOLD et al.:<br>"A rapid solid phase enzyme-linked binding assay for screening monoclonal antibodies to cell surface antigens."<br>see abstract<br>--- | 7-10 |